

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Christopher Robin Lowe *et al*
Serial No. : 10/520323
Filed : January 8, 2005
Art Unit : 1657
For : MONITORING OF CELLS

Commissioner for Patents
P O Box 1450
Alexandria, VA 22313-1450

DECLARATION OF ROBERT EDWARD PERRY

Sir:

I, Robert Edward Perry, make this declaration based on my personal knowledge and belief:

1. I am a European patent attorney with the firm Gill Jennings & Every LLP located in London, England. The firm represents the true assignee of this Application (Smart Holograms Limited) in intellectual property matters.
2. On May 20, 2002, I attended a meeting with the three inventors to discuss a project which they described as "FOAC"; that is an abbreviation for Fermenter On A Chip. At the meeting, we reviewed *inter alia* some of the information to be found in the Exhibit accompanying the Declaration of Christopher Robin Lowe, Craig J. L. Gershater ("Gershater") and Colin Alexander Bennett Davidson ("Davidson") that has previously been filed in connection with this Application ("The Lowe Declaration"). The inventors had been working on the FOAC project for some time, and continued to work on it, as is evident from the monthly reports for May, June, July and August 2002, attached hereto as Exhibits A, B, C and D respectively.

3. I received instructions to file a patent application at the meeting on May 20, 2002. I worked diligently on the case subsequently, and drafted a patent specification within the following 4 weeks; I sent the draft to the inventors on June 17, 2002, attended a meeting with Davidson on July 3, 2002, sent a revised draft to him on July 5, 2002, and reviewed his comments and revised and filed the specification at the UK Patent Office on July 9, 2002.

4. The Lowe Declaration was signed by 2 of the inventors, but not by Gershater. In an effort to locate him, I have spoken to Davidson and learned that he no longer works for the company that had contracted him to do the relevant work at the time of making the invention (GlaxoSmithKline plc), and has moved from his last known address, i.e. Cambridge Bioprocess Management Ltd., 68 London Road, Harston, Cambridge, CB2 5QJ, England, and is now resident elsewhere in Cambridge, England. Neither Davidson nor I has been able to contact him. I have conducted internet searches, but found nothing, including no entry for Gershater in the Cambridge area telephone directory.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

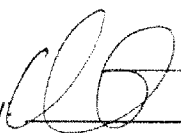
By:  _____ Date: August 27, 2009

Exhibit A

Fermenter On A Chip

Monthly Report for May 2002

(Work period 1/4/02-30/4/02)

Executive Summary

- Development of PDMS topcoating technique for FOAC chips
- Adhesion of bacteria to PDMS has been investigated
- PDMS film was used as a top coat in A3 chips for growth experiments with *Lactobacillus casei*

Future Work

- Application of PDMS film in chips for FOAC device
- Chemical modification of PDMS to reduce fouling and to allow for attachment of holograms

Development of PDMS top-coating techniques

Recently PDMS (poly-dimethylsiloxane) has become increasingly popular in microfluidics. Recent studies have highlighted methods for the rapid production of microfluidic prototypes in this material.

PDMS is commonly used in the electrical industry, referred to as 'silicone elastomer'. It is a rubbery, optically clear plastic that can be readily moulded to produce high resolution microfluidic devices. It is, however, highly hydrophobic and somewhat difficult to stick to other surfaces except in that it will bind hydrophobically to glass or plastic surfaces.

Due to the current production protocol for FOAC chips, it would be useful to directly laminate a top coat onto the chips without the need for printing an adhesive coating. By using a hydrophobically bonded plastic as opposed to a glued material there is also the advantage of being able to peel back the plastic to incorporate magnetic stirrers, filters, and to allow easy access to the growth medium.

To work out a protocol for coating FOAC chips with PDMS, clear silicone elastomer mix was obtained from RS. Elastomer was mixed with hardener at a ration of 10:1 and initially de-gassed in a vacuum to remove air bubbles, although subsequently centrifugation of the mix was found to be more convenient. Thin film of PDMS was prepared by pouring the mix onto LDPE sheet (a 'low energy' plastic with which the PDMS would not react) and heating at 80°C. The partially cured film was removed after various time periods and printed, un-laminated epoxy-chips were placed on the film.

Curing was then completed for 1 hour, the LDPE removed, and the hydrophobic seal on the chips tested by dispensing 2% crystal violet dye through them.

While all of the chips showed good sealing properties, the quality of the seal was not as good as that reported in other studies. In principle it should be possible to cure the PDMS to a surface to ensure a good seal.

To that end, partially cured PDMS was also bonded to glass and silanised glass, but without any increased level of bonding being evident. Varying the proportions of polymer with hardener also had no measurable effect.

To improve the curing protocol a different silicone elastomer mix was purchased, namely Sylgard 184 (Dow Corning). This material cures over a longer time at low temperatures, and can be cured rapidly at high temperatures allowing a complex assembly protocol to be developed with partial curing followed by adhesion.

Using the above protocol, optimum curing time to allow adhesion to the epoxy at 60°C was found to be 4 minutes 30 seconds, followed by further curing at 60°C for 10 minutes. However, significant smearing of the PDMS into the channels was observed. More complete initial curing prevented this smearing, but more cured material was found to give no advantage in adhesion over simple hydrophobic attachment.

It can be surmised, therefore, that while PDMS can be bonded to printed epoxy by attachment of partially cured material, the level of under-curing necessary is too great to allow for tight bonding without significant loss of channel resolution.

Fully cured (Sylgard) PDMS was treated with an atmospheric plasma for 30 seconds, and bonded to printed chips. While the treated material bonded fairly strongly to other treated samples of PDMS and to the polycarbonate base of the chips, it didn't bond at all to the epoxy, making this treatment somewhat inappropriate.

Hydrophobic bonding is sufficient to stop maintain a good seal of the chips, so it was decided to use that to construct the chips at present, subject to further testing of PDMS for bacterial adhesion.

Adhesion of Bacteria to PDMS

Using PDMS as a top coating for FOAC chips is only viable if the model bacterial strains used do not stick to the PDMS.

Standard biofilm formation experiments were conducted on PDMS samples, alongside LDPE as a control to ensure the data remained comparable with earlier work (figure 1). Although the water droplet contact angle measurement of both films was identical (data not shown) there was a large difference between the RS and Sylgard PDMS films in terms of bacterial adhesion. Sylgard, the slower curing material, proved to be far less prone to fouling.

Assorted treatments of the film were attempted to limit bacterial adhesion, including acid etching, heat treatment, flame treatment and oxygen plasma but no long lasting method was found. Chemical modification of the surface will be explored.

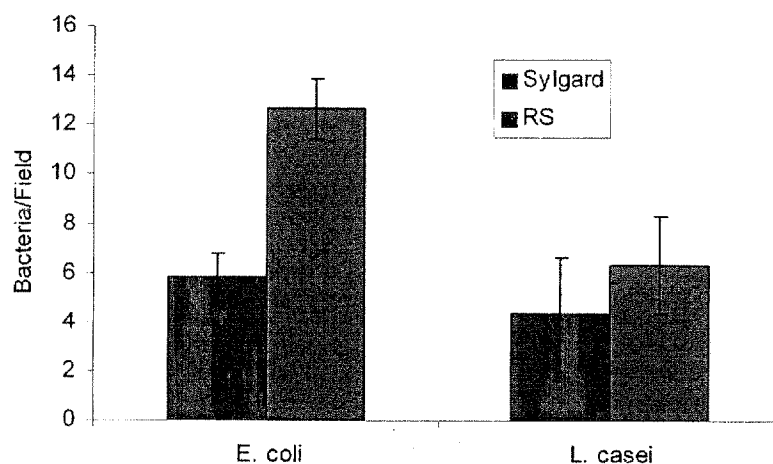


Figure 1: Attachment of *E. coli* and *L. casei* to PDMS

Use of PDMS for Top Coat of FOAC Chips

Sylgard was used to produce films as described above, which were in turn used as top-coats in FOAC. After production of the films careful selection was necessary, to rule out as the thin film LDPE used as a release film did not remain flat during polymerisation.

Lactobacillus casei was grown as described previously in a 2mm A3 chip for two hours. While there was some fouling of the PDMS surface after that time, the optical density of the culture doubled in 25 minutes, analogous to liquid culture.

Exhibit B

1. The first part of the document is a list of the names of the people who were present at the meeting.

2. The second part of the document is a list of the names of the people who were not present at the meeting.

3. The third part of the document is a list of the names of the people who were present at the meeting and who were also present at the meeting.

4. The fourth part of the document is a list of the names of the people who were present at the meeting and who were also present at the meeting.

5. The fifth part of the document is a list of the names of the people who were present at the meeting and who were also present at the meeting.

Fermenter On A Chip

Monthly Report for June 2002

(Work period 1/5/02-30/5/02)

Executive Summary

- Evaluation of spot holograms in chips in FOAC device
- Improvement of spot holograms in chips for FOAC device
- Development of high resolution optical features in PDMS

Future Work

- Further modification of holograms for FOAC device

High Resolution Optical Features in PDMS

The use of a mouldable, optically clear material in FOAC provides clear opportunity to produce high resolution optical features with a wide range of potential uses. By means of molding specific lenses in a fluidic device it would be possible to focus light in and out of sensing elements, allowing a reduction in size and therefore an increase in the number of sensors that can be placed on a surface.

A series of 2.5µm diameter lenses were constructed in PDMS. A hexagonal-array aluminium diffraction grating was used, and PDMS solution poured over the surface. After some optimisation of polymerisation, high resolution reproduction of the grating was achieved (figure 1).

The optical properties were found to be identical to those of the grating, in that light was diffracted in a hexagonal repeating pattern (figure 2), demonstrating that the hexagonal shape of the original is reproduced in the PDMS lenses, i.e. sub-micron level reproduction of the original grating. To the best of my knowledge, this is the highest resolution reported for PDMS lenses.

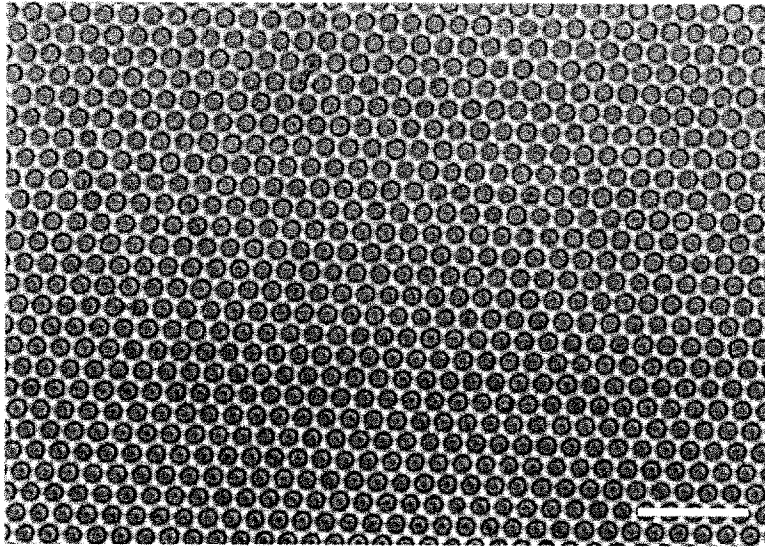


Figure 1: 2micron resolution reproduction of lenses in PDMS. Bar represents 10microns

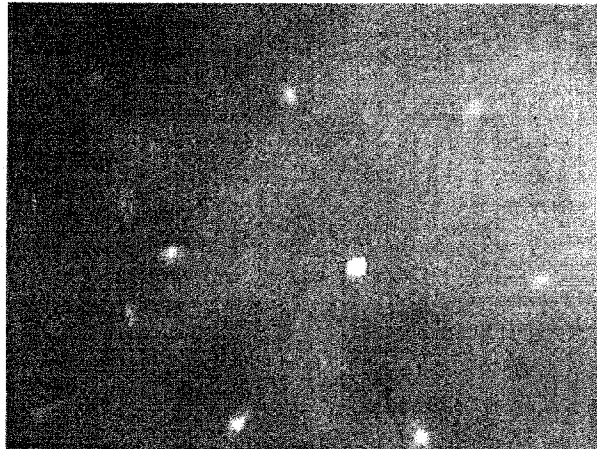


Figure 2: Red laser pointer light split into hexagonal repeating into pattern into a digital camera by PDMS grating

Chemical Modification of PDMS Surfaces

For incorporation as either a top or bottom layer in FOAC it would be useful to modify the surface to make it a less hydrophobic.

HEA, HEMA and MAA were reacted with PDMS surfaces in the presence of benzyl alcohol under U.V. Longer treatments, over 4 hours, successfully modified the surface making it much more hydrophilic. Further work will be necessary to optimise this system, the goal of which is to develop a means of selectively producing hydrophilic patterns on the PDMS and to allow the production of holograms on the surface.

Evaluation of Holograms in Chips in FOAC device

The first holograms in FOAC chips were evaluated at Diverse technologies. The holograms proved to be not bright enough to allow complete alignment to be completed.

Improvement of Holograms in Chips for use in FOAC device

The process of producing gelatine holograms in FOAC chips was re-evaluated. After re-optimising the silver-emulsion step (production of silver bromide from silver nitrate within the polymer matrix, with the addition of a sensitiser dye) and the development process (reduction of silver bromide to silver grains after exposure) much brighter holograms were produced. This process will now be repeated in spot holograms in chips to complete the alignment of the FOAC device.

Exhibit C

1. The first part of the exhibit is a list of the names of the persons who were present at the meeting on the 1st of June, 1964, at the home of Mr. and Mrs. J. W. Smith, 1234 Main Street, New York, New York.

Fermenter On A Chip

Monthly Report for July 2002

(Work period 1/6/02-30/6/02)

Executive Summary

- Various treatments of polyHEMA holograms have been investigated to reduce the swelling-dependent attachment of bacteria to the surface.
- PDMS has been further characterised for oxygen permeability, adhesion to fluidic chips and adhesion of bacteria
- Progress to date on developing holograms for use in FOAC is summarised
- Recent work on oxygen sensors for FOAC is summarised

Future Work

- Replication of gelatine holograms on PMMA to determine the nature of the optical problems
- Development of a polymeric matrix with high permeability for oxygen, possibly as an extension of previous perfluoromethacrylate polymer work
- Production of a holographic oxygen sensor with cobalt complex indicators.

Treatment of polyHEMA holograms for reduced attachment of bacterial cells to surface

Previous work has shown that the swelling/contraction of polyHEMA holograms can change the likelihood of biofilm initiation on the surface of the holograms, but not on the surface of identical polymers without holograms within them.

Assorted treatments of the holograms were investigated to prevent this, including modification of the fixing process (removal of unreacted silver bromide from the slides using sodium thiosulfite), replacing the silver grains with a thin coating of gold, and ultimately bleaching the silver with iodine in methanol to produce silver iodide grains. Of the methods investigated only silver iodide bleaching proved effective in reducing the swelling dependent attachment of bacteria to the surface of the holograms (figures 1 and 2).

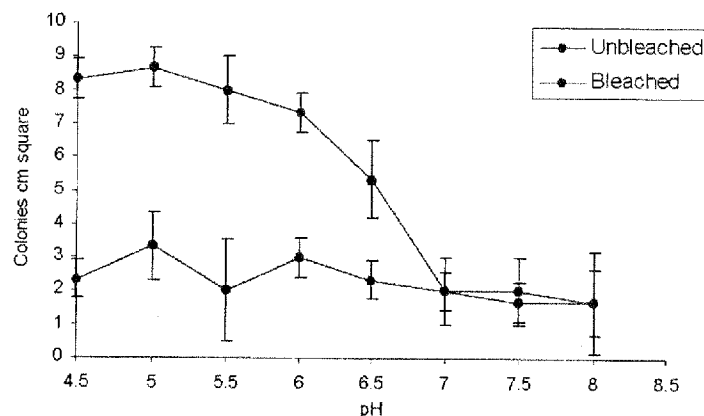


Figure 1: Reduction of adhesion of *E. coli* to 8% MAA polyHEMA hologram by bleaching with iodine

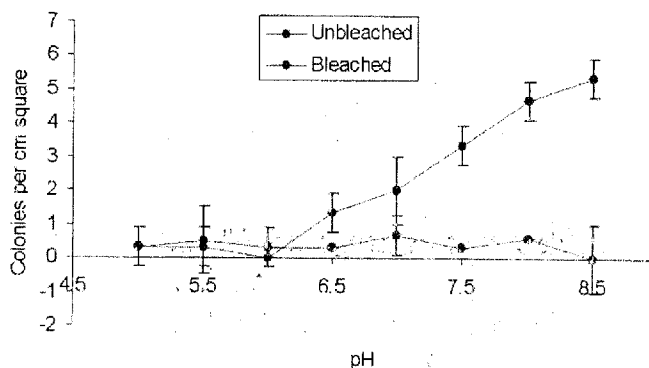


Figure 2: Reduction in adhesion of *E. coli* to 5% DMAEM polyHEMA hologram by bleaching with iodine

This result is difficult to explain in terms of a change in the surface properties of the polymer. It is possible that the silver iodide particles are less apparent on the surface of the polymer than the silver grains, thus forming an effectively smoother surface less amenable to attachment. It is likely that the silver or gold particles that are in the unbleached holograms provide an ideal surface for the attachment of the binding pilus of *E. coli*, with both metals being renowned for their capacity to bind to proteins.

By pre-bleaching holograms with iodine, they are not only less amenable to sticking by bacteria but they are also brighter. There is a resultant loss of useful life of the hologram, with the silver iodide being somewhat photo-active, and degrading in light to silver in time, but in a short lived disposable chip this should not be an issue.

Further Examination of Attachment of Bacteria to PDMS and Oxygen Permeability of PDMS

Chemical modification of PDMS was investigated to evaluate the effect on attachment of bacteria.

PDMS film reacted with benzyl alcohol and acrylate/methacrylate monomers with and without photoinitiator (DMPA) in UV. The result of this experiment on subsequent adhesion of *E. coli* to the surface of the PDMS is shown in figure 3.

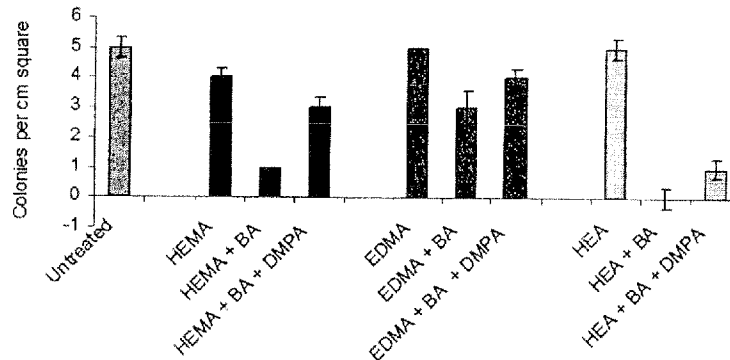


Figure 3: Effect of UV/acrylate treatment on attachment of *E. coli* to PDMS surfaces

As can be seen from the above figure, the most useful treatment to prevent the attachment of bacteria was to use either HEMA or HEA with benzyl alcohol, without any further initiator. PDMS thus treated also proved to be much more hydrophilic, and will be further investigated as a substrate for holography.

Some researchers have suggested that PDMS is more permeable to oxygen than we have previously thought. Thin (<50µm) film of PDMS has been produced by Meyer bar printing and used as a top coating for *E. coli* culture. Thus far the results suggest that this material is between LDPE and FEP in terms of oxygen permeability, making it much more oxygen permeable than we had previously thought, but further characterisation is needed.

Summary of Methods Used to Incorporate Hologram in FOAC Chip

Included for reference is a list of the technical issues involved in incorporating the hologram in the FOAC chip.

Initially, the spot holograms produced on PMMA were deemed to dry out too fast, so it was necessary to formulate a top-coat that could be applied at the IoB to maintain hydration. Application of TPX film was initially used to circumvent this problem.

The chips were, however, found to be exceedingly difficult to fill. Our initial assumption was that the main problem was one of surface hydrophobicity, but ultimately this was not sufficient to allow the chips to be easily filled without damaging the brittle epoxy print. It transpired that the epoxy print on PMMA was a lot more brittle than previous prints on PC, so it was decided for the purpose of finishing off the FOAC device to move to a removable top coat.

A PDMS top coat was produced to allow this, that top coat being bonded to the chip hydrophobically.

The alignment of the optics in the device proved to be a lot more damaging to the holograms than had been anticipated, meaning that the polymerisation procedure had to be re-addressed to produce more durable holograms. Unfortunately, the extra cross linking of the gelatine layer had a negative effect on the brightness of the hologram which had to be re-addressed in the hologram production process.

Brighter, more durable holograms in FOAC chips have thus far proved impossible to observe in the FOAC device, despite identical holograms on plain PMMA being relatively easy to observe. Currently we are re-evaluating holograms on the plain PMMA and comparing them with those in the chips to determine the nature of any difference between them.

Holographic Oxygen Sensor

Introduction

Many oxygen sensors have been developed and used widely in many ways, such as in the environment and in medicine. They can be divided into two kinds, optical and electrochemical sensors with the optical sensors being divisible into fluorescent, luminescent and phosphorescent sensors. The typical electrochemical sensor is Clark electrode. Of the two broad categories, the optical sensor is much more sensitive than the electrochemical sensor, but the low specificity (for example fluorescent interference from other materials present) may be troublesome, especially in biological applications.

Aim of the project

The aim is to extend the preliminary work already done on a holographic technique to produce a convenient, simple and cheap oxygen sensor.

For the oxygen sensor, there are three key necessities:

- 1) Indicator which reacts with oxygen;
- 2) Polymer which has high permeability for oxygen;

- 3) Integration into a hologram of the indicator and oxygen permeable matrix.

Indicator

Although many oxygen sensors have been suggested, few indicators are used in the sensors:

- 1) Oxygen carriers, such as haemoglobin and perfluoro compounds;
- 2) Metal complexes, which react with oxygen reversibly, such as Ru and Os complexes. They are widely used in optical oxygen sensors;
- 3) Natural enzymes, such as glucose oxidase.

After a review of the literature, we choose a cobalt complex as our first oxygen sensor indicator. This complex is a useful compound because it reacts with oxygen reversibly both in solution and solid phase, the details of mechanism being shown in figure 4.

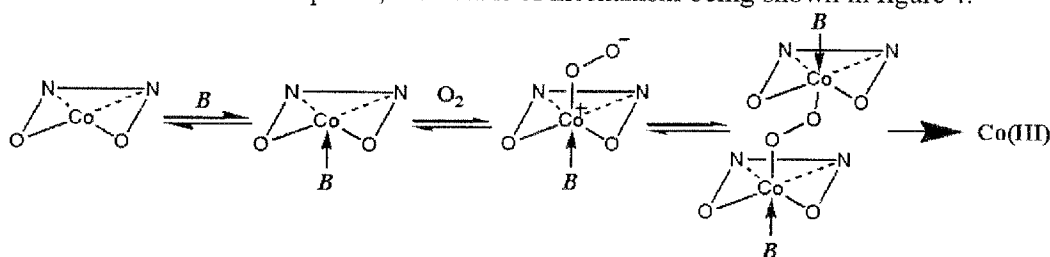


Figure 4: Reversible oxygen binding to cobalt complex.

According to the mechanism of the above cobalt complex, we designed and synthesized a five-coordinated cobalt complex, starting from aldehyde, diamine and cobalt chloride, producing first a four-coordinated complex. This was followed by adding pyridine to produce a five-coordinated cobalt complex.

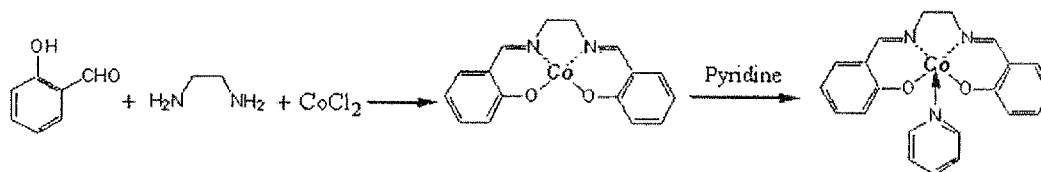


Figure 5: Synthesis of five co-ordinated cobalt complex

Figure 6 shows UV-data for the five-coordinated cobalt complex reacting reversibly with oxygen by bubbling oxygen and nitrogen through the cuvette. Figure 7 shows that the reaction of the complex with oxygen is reversible (following the initial reaction of the complex which causes an irreversible change).

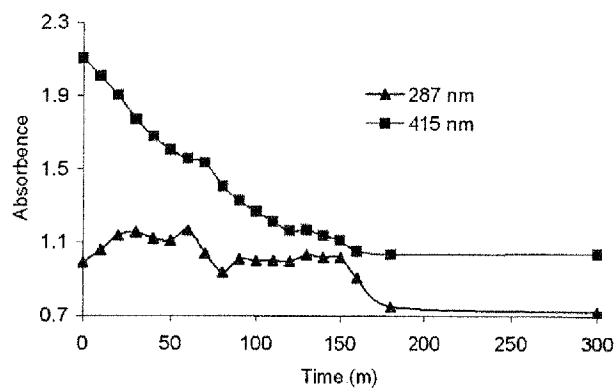


Figure 6: Change in absorbance at 287nm and 415nm of cobalt complex in solution following sparging of 50ml of oxygen.

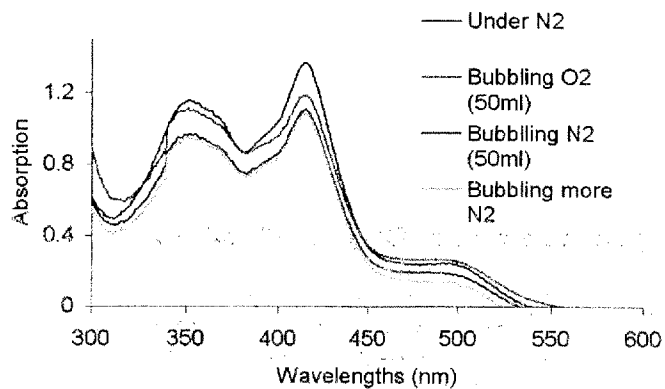


Figure 7: Effect of successively sparging 50ml samples of oxygen and nitrogen through solution of cobalt complex.

Exhibit D

Fermenter On A Chip

Monthly Report for August 2002

(Work period 1/7/02-30/7/02)

Executive Summary

- Replication of gelatine holograms on PMMA to determine the nature of the optical problems
- The procedure for producing polyHEMA holograms on PMMA has been investigated for potential streamlining
- Software for the FOAC device has been refined to remove background noise
- Further development of glucose sensor

Future Work

- Development of a polymeric matrix with high permeability for oxygen, possibly as an extension of previous perfluoromethacrylate polymer work.
- Production of a holographic oxygen sensor with cobalt complex indicators.
- Evaluation of need for further hardware modifications in FOAC device.

Gelatin Holograms on PMMA

As has been documented, a serious problem in recent months has been the replicability and durability of holograms on PMMA. To address this issue, the pre-treatment of PMMA surfaces has been examined in great depth.

A range of treatments of the surface have been re-visited and re-optimised, including heat treatment, flame treatment, acid treatment and reaction of the surface with HEA and HEMA. No improvement of attachment of gelatin to the surface was found in any of these treatments, and consequently no improvement in brightness or durability of the holograms ensued.

Pre-treatment of the PMMA with the poly-amide Ultramid was re-optimised. Use of a less volatile chlorinated solvent in the treatment was found to produce a much more uniform treatment with much less light scattering on the surface, ultimately producing a brighter and more durable hologram. Such holograms were evaluated in the FOAC device, but were still found to have too much associated light scattering to be valuable. A further option of modifying the FOAC device with extra filters to allow this generation of holograms to be used is under consideration.

PolyHEMA holograms on PMMA

The method previously developed for producing polyHEMA holograms on PMMA is long winded and overly complex, with much scope for improvement. The method currently used is to cross-link a thin film of gelatin on the surface of the PMMA with both dichromate and glutaraldehyde, followed by polymerisation of the polyHEMA on the surface of the gelatin.

As the diffractive indexes of PMMA and polyHEMA are similar, it should be possible to achieve improvements in adhesion by 'roughing up' the surface of the PMMA. Initial attempts to achieve this with mechanical abrasion of the surface were successful, but no mechanism to produce sufficient roughness without affecting the optical properties of the plastic was found. A second technique of etching the surface with a solvent has been evaluated, and while this has had some success it is hard to produce an even surface treatment by this technique. Damage caused to the interface of the PMMA and the polyHEMA by the hologram development process has been much reduced by application of techniques developed for producing gelatin holograms on PMMA.

A further improvement in the process might be achievable by using a heat treatment subsequent to polymerisation of the polyHEMA on the surface of the PMMA, this will be subject to further investigation.

PolyHEMA holograms on PDMS

Previous work demonstrated the possibility of modifying the surface of PDMS by imprinting with micro-lenses. PDMS patterned with 2.5µm micro-lenses was further treated with HEA, benzyl alcohol and DMPA as previously described prior to polymerisation of 6%MAA polyHEMA on the surface. Even polymerisation was achieved, with some evidence of the patterning on the surface of the PDMS apparent after polymerisation of the polyHEMA. A hologram was developed in the polyHEMA, and while there was some loss of polyHEMA from some regions the hologram was bright and useable. Further development of this procedure will follow, in the hope that this procedure will allow the production of holograms on the surface of PDMS in FOAC.

Software for the FOAC device has been refined to remove background noise

Software for use with the FOAC device has been improved to remove a great deal of background noise that was limiting the use of some weaker holograms. An algorithm has been added to the software that corrects for background noise. Figure 1 shows outputs from a hologram before and after correction.

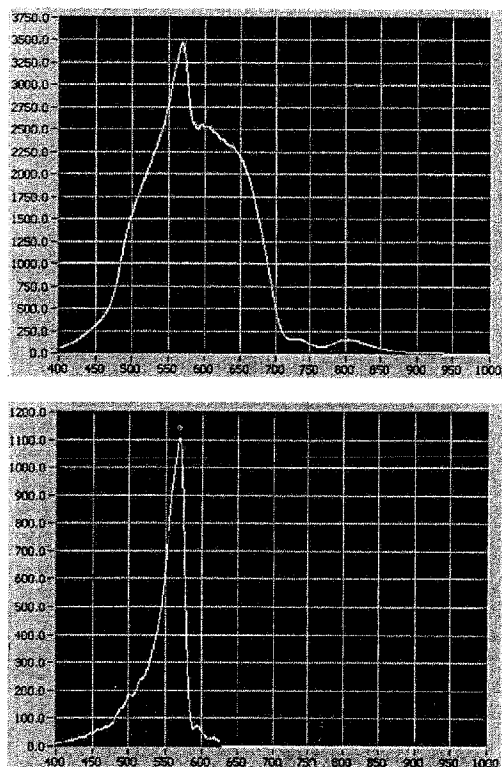
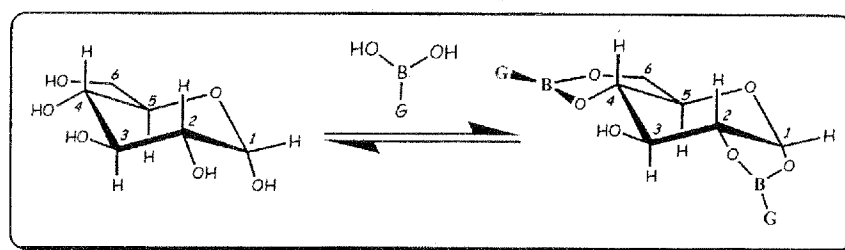


Figure 1: Software correction to remove background noise from hologram reading in FOAC device. Uncorrected data shown top, corrected data below.

Holographic Glucose Sensors

Work is ongoing at the IoB to develop a holographic glucose sensor. The main associated challenge is to design and synthesize glucose receptors. According to the literature, simple boronic acids readily and reversibly form cyclic esters with monosaccharides in aqueous basic media, the most common interaction for glucose being with 1,2- and 4,6-diols to form five- and six-membered rings via two covalent bonds (see following scheme 1):



For our application, it is required that the receptors will bind with glucose in acidic or neutral media because cell growth sera and microbiological media are rarely sufficiently basic. Previously, little on this topic has been published.

Targets

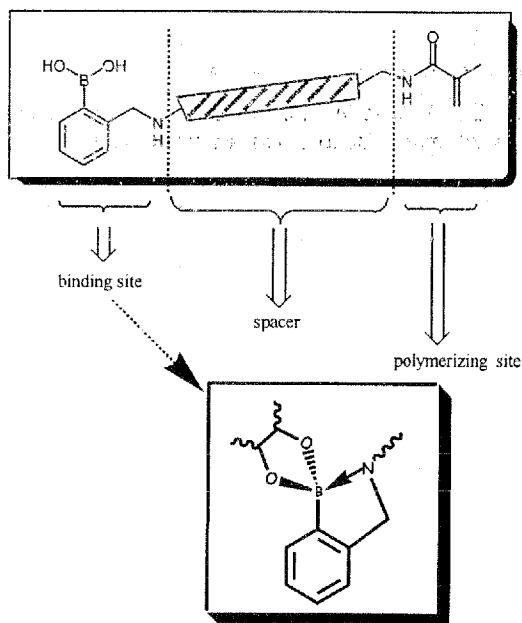
- 1) To design and synthesize receptors which bind with glucose in aqueous acidic media;
- 2) To detect a binding ability of the receptors in solution and hologram both.

Synthesis

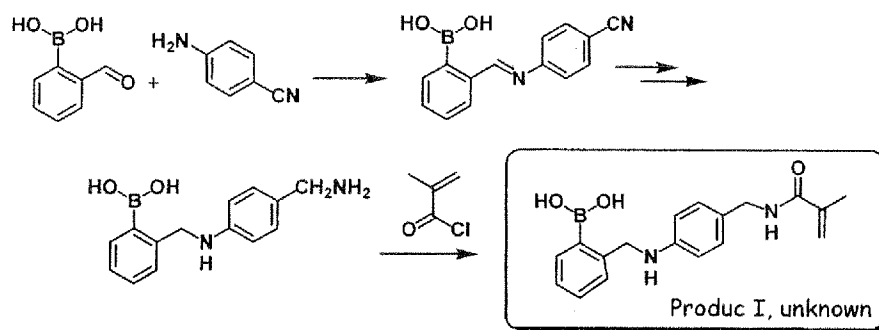
For the receptors, there are three parts included:

- 1) a binding site which reacts with glucose;
- 2) a polymerising site which introduces the binding site into polymers;
- 3) a spacer which links the binding and polymerising sites together.

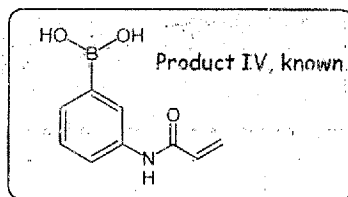
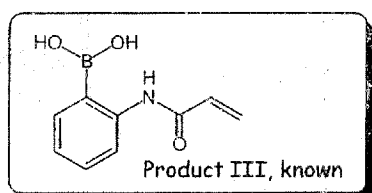
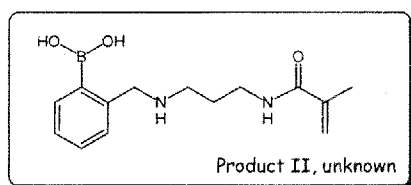
The following scheme II shows a basic structures for our target molecules.



So far, four boronic acids have been synthesized, two of them being novel compounds. The details for synthesis are presented here.



The others were made by the similar method, their structures show here.

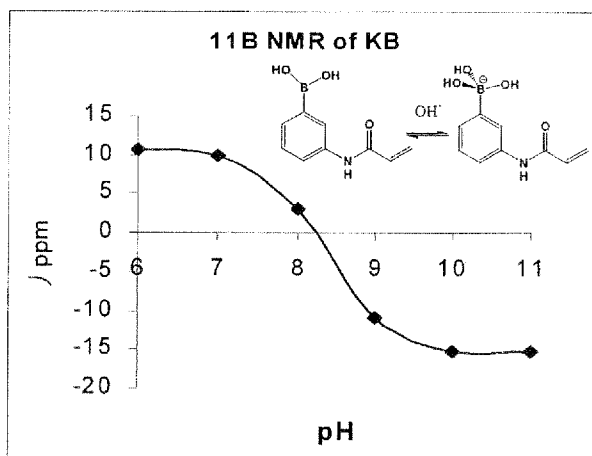


The structures of all products were characterized by NMR and MS.

The Binding Ability

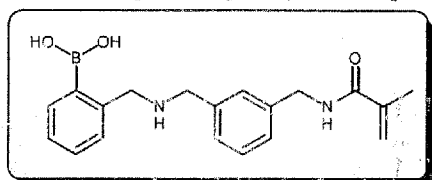
The binding studies of the receptors have only recently commenced, but results to date are highly promising.

The following ^{11}B NMR data shows, for product IV, boron geometry is dependent on pH, as $\text{pH} < 7.0$, the main geometry of boron is triangular, its ^{11}B NMR δ (ppm) = ~ 10.0 ; as $\text{pH} > 10.0$, tetrahedral style is dominated, its ^{11}B NMR δ (ppm) = ~ -15.0 . Between pH 7.0 and 10.0, there is an equilibrium. According to the data, it's possible for us to use this receptor (product IV) binding with glucose between pH 7.0~8.0.



Future Work

- 1) We are planning to synthesis product V, (below).



- 2) We are continuing binding studies for other receptors, and also planning to detect a binding ability with monosaccharides by NMR. These investigations are very important and helpful for us to put the receptors into holograms.